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Simvastatin Attenuates Formalin-Induced Nociceptive Behaviors by Inhibiting Microglial RhoA and p38 MAPK Activation

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Abstract: **Several recent studies have revealed that statins exert anti-inflammatory effects in addition to their lipid-lowering property in vivo and in vitro. Recently, statins were shown to alleviate pain associated trauma in a neuropathic pain model. The aim of the present study was to investigate the underlying mechanisms of analgesia caused by the lipophilic statin simvastatin in an animal model of formalin-induced pain in the rat. Intrathecal pretreatment with simvastatin significantly attenuated the second phase of the acute nociceptive response to formalin injection, and daily administration of simvastatin for 7 days inhibited the long-term mechanical hyperalgesia caused by formalin injection. Spinal microglial activation (detected by Iba-1 and CD11 b immunohistochemistry and Western blot), and phosphorylated-p38 mitogen-activated protein kinase (detected by immunohistochemistry and Western blot) were significantly inhibited by simvastatin treatment at day 7 after formalin injection. In addition, peripheral formalin injection induced a significant increase in microglial RhoA activation (detected by membrane RhoA translocation ratio using Western blot) in the spinal cord. The spinal RhoA activation in microglia was reversed by simvastatin treatment. These findings suggest that simvastatin attenuates formalin-induced nociceptive behaviors, at least in part, by inhibiting microglial RhoA and p38 mitogen-activated protein kinase activation.**

Perspective: ***Our novel findings indicated that simvastatin attenuated formalin-induced nociceptive responses by inhibiting microglial RhoA and p38 mitogen-activated protein kinase activation. Inactivation of RhoA-p38 signaling pathway may be a pharmacologic target for treating microglia-directed central nervous system inflammation and chronic pain conditions.***

Key words: *Formalin pain model, microglia, p38 mitogen-activated protein kinase, RhoA, statins.*

Statins are selective inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis.[22] Besides their broad use as lipid-lowering drugs, recent reports have revealed immunomodulatory and anti-inflammatory activities for statins in vivo and in vitro.[3,8,39,47] These properties may be relevant to the statins' therapeutic effects in neuroinflammatory conditions.[21,39,48] Currently, statins are being tested for their potential efficacy in treatment after brain injury. The treatment inhibited a number of inflammatory processes known to be important in brain damage and suppressed the secretion of potentially damaging cytokines such as interleukin-1β and tumor necrosis factor-alpha in spinal cord injury and ischemic stroke.[1,5] Treatment with atorvastatin and simvastatin markedly reduced functional neurologic deficits after traumatic brain injury in rodents. In this study, reductions in neurologic deficits were accompanied by the suppression of inflammatory cytokine mRNA expression in the brain parenchyma.[45] Most recently, rosuvastatin was tried in humans and it was found that the treatment could induce an anti-inflammatory effect and promote recovery after traumatic brain injury in a clinical trial.[34]

Neuropathic pain has many features of a neuroimmune disorder. Glia are known to be activated by nociceptive events and play important roles in the generation and maintenance of pain hypersensitivity.[10,11,18,20,27,43,46] Activated microglia and astrocytes are the main sources of inflammatory mediators and

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cytokines in the central nervous system (CNS). Several reports have demonstrated that statins may exert anti-inflammatory action by a way of inhibiting the activation of microglia and astrocytes.[9,23,26] Most recently, systemic administration of statins was shown to block the development of mechanical allodynia and thermal hyperalgesia in neuropathic pain models.[6,36] Additionally, the statins also significantly reduced the spinal microglial and astrocyte activation produced by sciatic nerve injury.[36]

Although statins have been effective in treating nerve-injury animals, little is known regarding the mechanisms underlying these promising effects of statins. Recent reports suggested that statins exerted anti-inflammatory actions through their ability to prevent the isoprenylation of members of the Rho family of small G proteins, resulting in the functional inactivation of these G proteins.[8,42] RhoA, a small GTPase, is a member of a family of small molecular G proteins, which are involved in many cellular functions, including cytoskeletal rearrangement, cell motility, phagocytosis, intracellular trafficking, transcriptional regulation, and cell growth and development.[40]

In the present study, we investigated the anti-hyperalgesic effects of intrathecally applied simvastatin on formalin injection–induced nociceptive behavior and microglial activation in rats. To determine whether statins function by inhibiting microglial activation, immunohistochemical staining and semiquantitative Western blot analysis using Iba-1, CD11 b, mitogen-activated protein kinases (MAPKs), and membrane RhoA expression in the spinal cord were used. This is the first report indicating that peripheral formalin injection induces an increase in spinal microglial membrane RhoA translocation (active state) ratio that can be reversed by simvastatin treatment.

Methods

Animals and Treatments

Male adult Sprague Dawley rats weighing 200 to 225 g (Vital River Laboratory Animal Technology Co. Ltd, Beijing, P R China) were used, and all protocols for the experiments were approved by the Animal Care and Use Committee of Peking University Health Science Center and certified that the care and use of animals conformed to applicable national/international guidelines. All rats were housed at 23°C (±1°C) on a 12-hour light/dark cycle with free access to food and water.

All nociceptive testing and collection of data were done by personnel blinded to the treatment of the animal.

Chronic lumbar intrathecal (i.t.) catheters were implanted according to the procedure previously described.[38] Briefly, under adequate anesthesia with sodium pentobarbital (40 mg/kg, intraperitoneal [i.p.]), a polyethylene catheter (PE-10, 20 cm; Warner Instruments, Hamden, CT) was introduced in the subarachnoid space via the L5/6 intervertebral space and advanced rostrally 3.0 cm in order to reach the lumbar enlargement. The catheter was sutured to the fascia, tunneled subcutaneously on the back of the rats, and its proximal end was externalized in the occipital region. Animals were allowed to recover for at least 5 days after implantation, and rats that had signs of neural dysfunction were removed from the study (2 rats in the study).

Simvastatin (Sigma-Aldrich, St. Louis, MO) was dissolved in 90% sterile saline, 5% dimethylsulfoxide, and 5% Cremophor EL (Sigma-Aldrich).[29] In behavioral experiments, we tried several concentrations of simvastatin (.1, 1, 10, and 20 μg/10 μL). The results indicated that 10 μg was the ideal dose for the subsequent protein-semiquantitative experiments. The maximal concentration (20 μg) did not show a reversal effect on spontaneous nociceptive behaviors, and this extremely high concentration of drug may produce adverse reactions such as toxicity.

Rats were randomly divided into 3 groups: naïve, in which rats received saline injection into the rat hind paw instead of formalin injection; saline control group (vehicle), in which rats were given vehicle (10 μL) intrathecally including 90% sterile saline, 5% dimethylsulfoxide, and 5% Cremophor EL before hind paw formalin injection; and simvastatin-treated group (simvastatin), in which simvastatin solution (10 μL) was delivered intrathecally and the catheter flushed with 10 μL of saline. Simvastatin (.1, 1, and 10 μg) or vehicle was delivered intrathecally 30 minutes prior to subcutaneous injection of 100 μL 5% formalin into the plantar surface of the right hind paw at day 0. Further simvastatin injections were subsequently repeated once daily at 3 PM until animals were sacrificed at day 7. In previous reports, statins were generally delivered by oral or intraperitoneal administration. We injected simvastatin intrathecally to observe a direct and region-limited activity on the CNS, to exclude the possible action of simvastatin on injured peripheral tissues.

Assessment of Acute Spontaneous Nociceptive Responses

Each rat (6 rats in each group, total 24) was habituated in a 30 × 30 × 30-cm clear plexiglass chamber for at least 1 hour for 3 days to familiarize them to the surroundings. On the test day, following 100 μL 5% formalin injections, rats were returned to the test chamber immediately. Nociceptive responses (flinches and licking) were recorded by a video camera for 60 minutes, and counted for each 5-minute block. Nociceptive responses were collected for phase I (0–5 minutes) and phase II (20–40 minutes) and compared among groups.[11] Simvastatin (.1, 1, and 10 μg) or vehicle solution was administered by the i.t. route 30 minutes prior to subcutaneous injection of formalin.

Assessment for Long-Term Mechanical Nociceptive Thresholds

The mechanical thresholds for nociceptive responses were measured as described previously and all tests were conducted before treatment and on days 1, 3, 5, and 7 before drug delivery that day.[32] Briefly, the rat was habituated to standing on its hind paws and against the tester's (X.-Y.C.) gloved hand. The test consisted of

evoking a hind paw flexion reflex with a hand-held force transducer (electronic anesthesiometer; IITC Life Science, Woodland Hills, CA) adapted with a .5-mm^2 polypropylene tip. The area tested was the dorsal surface (formalin injections were in the plantar surface) of the right hind paw, between the third and fourth metatarsals. The investigator was trained to apply the tip perpendicular to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the withdrawal of the paw followed by clear flinching behavior, repeated 3 times for each animal in 1-minute intervals. The mean force value of all 3 applications was used as the withdrawal threshold. The percentage change from the baseline threshold was calculated for each time point for each rat.

Immunohistochemistry

Animals, including naïve, vehicle, and simvastatin (10 μg) groups (2–4 rats for each group), were anesthetized on day 7 after formalin injection with an overdose of pentobarbital sodium (100 mg/kg, i.p.) and euthanized by transcardiac perfusion with 250 mL of body-temperature, .1 M phosphate-buffered saline (pH 7.4), followed by 300 mL ice-cold 4% paraformaldehyde/4% sucrose in .1 M phosphate buffer, pH 7.4. After perfusion, the lumbar spinal cord (L4–5) was removed, postfixed in 4% paraformaldehyde fixative for 4 hours, and then placed in 30% sucrose solution (in .1 M phosphate buffer) over 2 nights at 4°C. Thirty-micrometer-thick spinal cord sections were cut transversely on a cryostat for free-floating immunohistochemical staining. The sections were blocked with 4% normal goat serum (NGS) and then incubated for 48 hours at 4°C with primary antibody. Binding was visualized with FITC-conjugated secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Stained sections were examined with a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan), and images were captured with a CCD spot camera (Olympus DP71). For double immunofluorescence, the sections were incubated with a mixture of 2 primary antibodies from different species. Binding was visualized with FITC-conjugated and TRITC-conjugated secondary antibody. The primary antibodies used in the present study were as follows: Iba-1 (microglia marker, 1:200; Wako Chemicals, Osaka, Japan), CD11 b (microglia marker, 1:200; Serotec, Oxford, UK), phospho-p38 (1:200; Cell Signaling Technology, Beverly, MA), NeuN (neuronal marker, 1:5,000; Chemicon Temecula, CA), glial fibrillary acid protein (GFAP, astrocyte marker, 1:200; NeoMarkers, Fremont, CA), and RhoA (1:200; Abcam, Cambridge, MA).

Western Blot Analysis

Animals from naïve, vehicle, and simvastatin (10 μg) groups (6 rats for each group) were deeply anesthetized at day 7 after formalin injection and then decapitated. The spinal cord segments L4–5 (lumbar enlargement) ipsilateral to the injection were removed quickly and homogenized in ice-cold RIPA buffer (Cell Signaling Technology; supplemented with 1 mM phenylmethylsulfonyl fluoride, phosphatase and protease inhibitor cocktail; Sigma-Aldrich). The homogenate was centrifuged at 15,000 × g for 40 minutes at 4°C. The protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Aliquots (30 μg protein) were subjected to 12% SDS-PAGE and transferred electrophoretically to PVDF filters (Millipore, Bedford, MA). The PVDF membrane was blocked with 5% nonfat milk in Tris-buffered saline containing .1% Tween-20 for 1 hour in room temperature. The following antibodies were used to detect proteins: anti-RhoA (1:1,000; Santa Cruz), anti-Iba-1 polyclonal antibody (1:1,000; Wako), anti-CD11 b (1:1,000; Abcam), anti-phosphor-p38 (1:1,000; Cell Signaling Technology), anti-phosphor-Erk (1:1,000; Cell Signaling Technology), and mouse polyclonal antibodies against p-JNK (1:1,000; Cell Signaling Technology). After washing, the antibody–protein complexes were probed with HRP-conjugated secondary antibody (1:5,000; Jackson ImmunoResearch), developed in ECL solution for 3 minutes, and exposed onto Kodak hyperfilms (Kodak, Xiamen, China). The intensity of immunoreactive bands was quantified using NIH ImageJ 1.38 software (US National Institutes of Health, Bethesda, MD), normalized to the density of internal control β-actin (1:1,000; Santa Cruz), and expressed as -fold changes as compared to vehicle group.

Separation of Membrane Localized RhoA

The separation of membrane localized RhoA was performed by using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo, NO.89826; Pierce Biotechnology, Rockford, IL). The spinal cord segments L4–5 ipsilateral to the injection were removed quickly after decapitation of rats (4 rats for each group) and homogenized in ice-cold Tris-buffered saline (.025 M Tris, .15 M NaCl; pH 7.2). The homogenate was centrifuged at 1,000 × g for 5 minutes at 4°C. The supernatant was discarded and pellet resuspended in 150 μL Mem-PER Reagent A (Pierce Biotechnology). After 10 minutes, 450 μL of diluted Reagent C (C:B = 2:1) was added to each tube of lysed cells and tubes put on ice for 30 minutes, vortexing every 5 minutes. Then tubes were centrifuged at 10,000 × g for 3 minutes at 4°C. Supernatant was put in new tubes and incubated for 10 minutes in a 37°C water bath to separate the membrane protein fraction. The tube was then centrifuged at room temperature for 2 minutes at 10,000 × g to isolate the hydrophobic fraction from the hydrophilic fraction. The majority of membrane protein was in the lower hydrophobic fraction, which was analyzed by Western blot as mentioned above. The hydrophilic phase was carefully removed from the hydrophobic protein phase and transferred to a new tube for cytosolic protein analysis by Western blot.

Statistical Analysis

All data were presented as mean ± standard error of the mean. Statistical significance was determined by 1-way analysis of variance for repeated measures followed by Dunnett's t-test in behavioral analysis, and by unpaired t-test for Western blot densitometry analysis using

SPSS software (version 16.0; SPSS Inc, Chicago, IL). These evaluations were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA). In all analyses, a P value < .05 was considered statistically significant.

Results

Intrathecal Administration of Simvastatin Decreased Formalin-Induced Phase II Spontaneous Nociceptive Behaviors

Rats injected with 5% formalin displayed classic biphasic spontaneous nociceptive behaviors immediately after injection, lasting for 1 hour. In this study, we quantified the number of flinches and the duration time of licking behavior per 5 minutes within 1 hour in the 4 groups (.1, 1, and 10 μg simvastatin-injected groups and saline-injected control; see Fig 1 A). The spontaneous nociceptive behaviors were divided into 2 clear-cut phases: phase 1 (0–5 minutes) and phase 2 (20–40 minutes). The i.t. injection of simvastatin produced a dose-dependent reduction of flinching behaviors in phase 2, whereas no reduction was found in phase 1 (Fig 1B). There was no significant change in licking responses either in phase 1 or in phase 2 (data not shown).

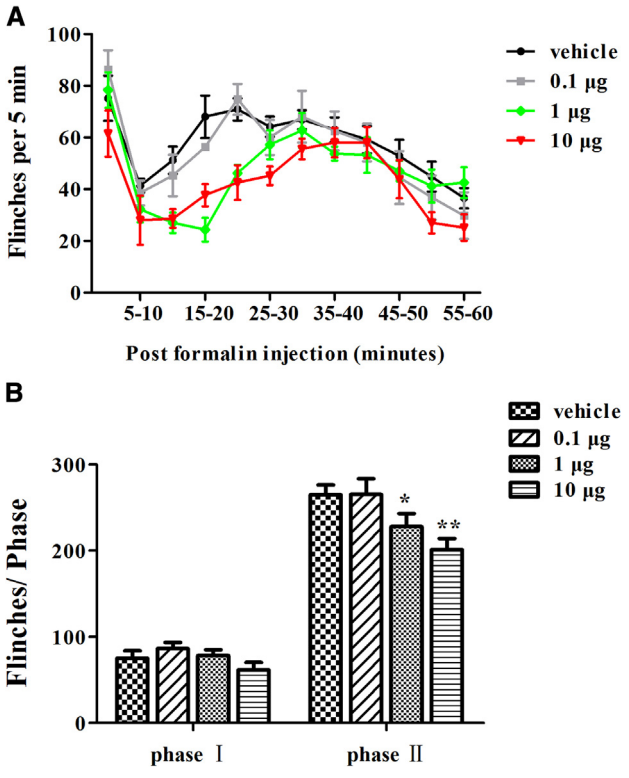

Figure 1. Effect of i.t. pretreatment with simvastatin on formalin-induced spontaneous nociceptive behavior. **(A)** Time course of flinching behavior following peripheral 5% formalin injection 30 minutes after intrathecal administration of vehicle or .1, 1, and 10 μg of simvastatin. **(B)** Total numbers of flinches during phase I (0–5 minutes) and phase II (20–40 minutes) in animals treated with simvastatin or vehicle. Simvastatin at 1 and 10 μg significantly suppressed phase II flinching behavior. *P < .05, **P < .01, as compared with the vehicle group, n = 6.

Intrathecal Administration of Simvastatin Reduced Formalin-Induced Long-Term Persistent Mechanical Hyperalgesia

We have previously reported that formalin-injected tissue injury produces a third phase of nociceptive behaviors: secondary long-term persistent thermal and mechanical hyperalgesia lasting for about 3 weeks.[12] In the present study, we tested the mechanical nociceptive threshold on the dorsal surface (formalin was injected into the plantar surface) by using a .5-mm-diameter polypropylene rigid tip. Simvastatin (.1, 1, and 10 μg) or its vehicle was delivered once daily for 7 successive days prior to mechanical hyperalgesia testing. The administration of 1 and 10 μg simvastatin significantly reduced the formalin-induced persistent mechanical hyperalgesia at each time point (Figs 2A and 2B).

Intrathecal Administration of Simvastatin Attenuated Formalin-Induced Spinal Microglial Activation

Iba-1 and CD11 b immunohistochemistry have been widely used to identify microglia in the spinal cord. To

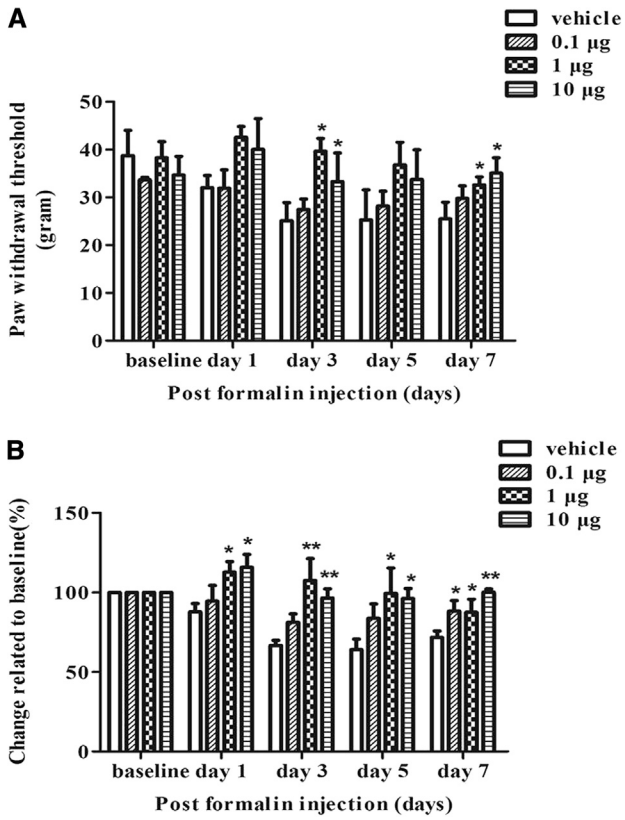

Figure 2. The effect of i.t. injection of simvastatin on formalin-induced long-term mechanical hyperalgesia. **(A)** The mean values of paw withdrawal thresholds with vehicle and 3 different concentrations of simvastatin at baseline before and days 1, 3, 5, and 7 after formalin injection. **(B)** The percentage changes from baseline threshold following formalin injection with and without simvastatin treatment. *P < .05, **P < .01, as compared with the vehicle-treated group at each point, n = 6.

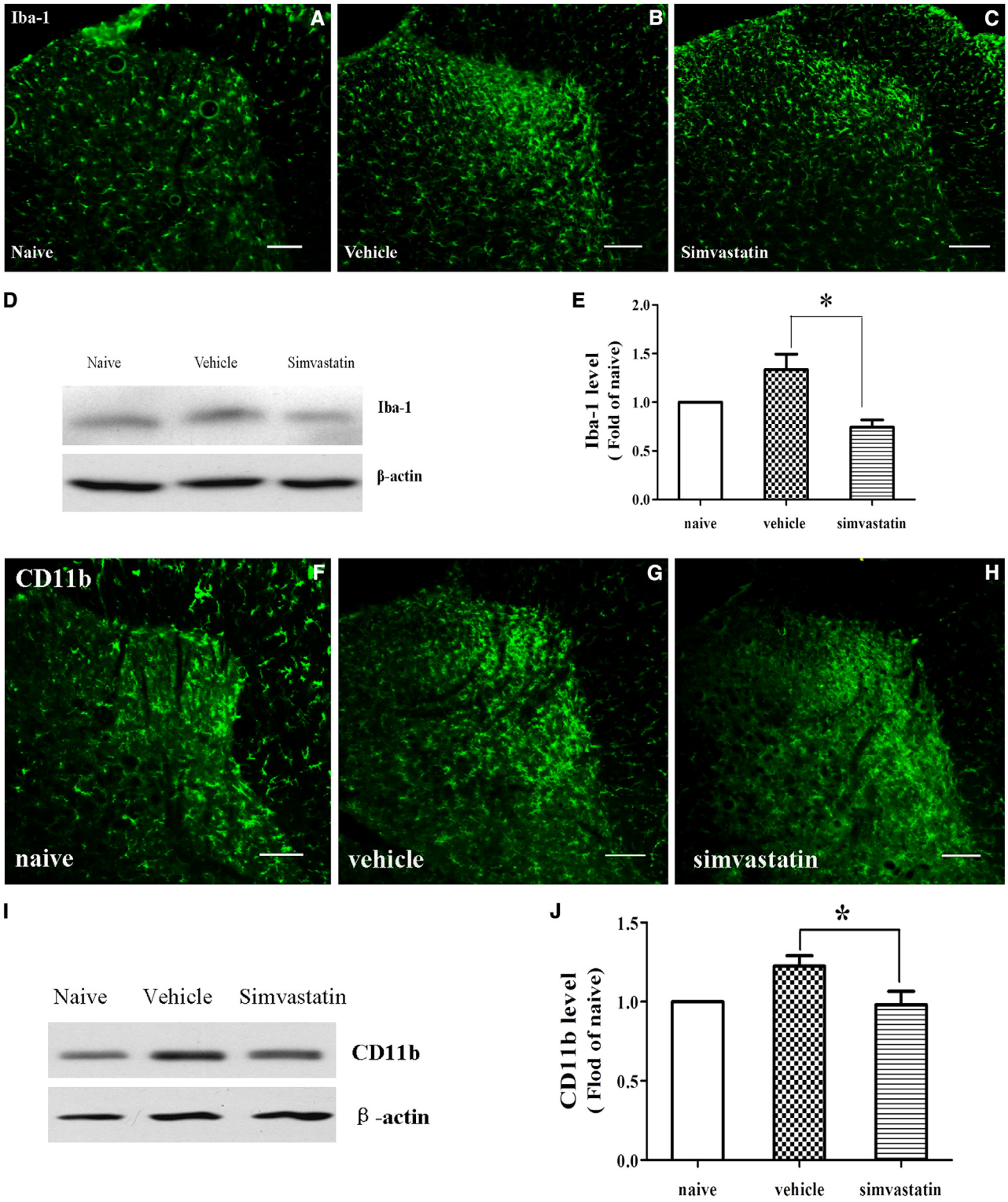

Figure 3. The effect of i.t. injection of simvastatin on formalin-induced microglial activation in the ipsilateral spinal cord at day 7 after 5% formalin injection. **(A–C)** Iba-1 immunoreactivity on the ipsilateral side of the dorsal spinal cord from naïve, vehicle-, and simvastatin-treated animals (scale bars: 100 μm). **(D** and **E)** Representative bands and quantification of Western blot analysis showed that simvastatin suppressed the increased Iba-1 protein level. **(F–H)** CD11 b immunoreactivity from naïve, vehicle-, and simvastatin-treated animals (scale bars: 100 μm). **(I** and **J)** Representative bands and quantification of Western blot analysis showed that simvastatin suppressed the increased CD11 b protein level. *$P < .05$, as compared with the vehicle group, n = 6.

detect the effects of simvastatin on the response of spinal microglia to the formalin test, both Iba-1 and CD11 b expression were measured by immunofluorescence and Western blot. Formalin injection into the plantar hind paw resulted in robust spinal microglial activation with morphologic changes characterized by

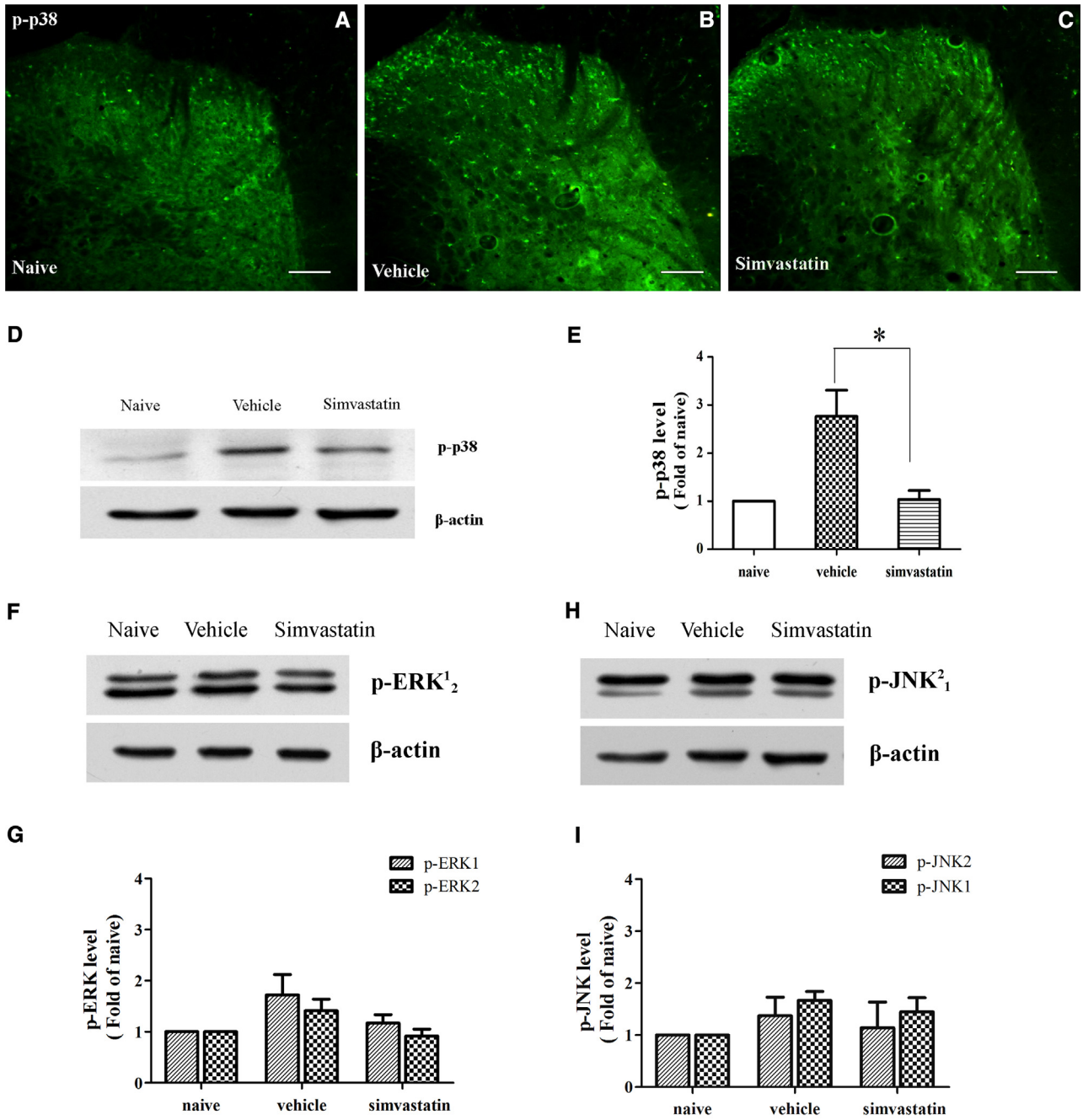

Figure 4. The effect of i.t. injection of simvastatin on spinal MAPK pathway at day 7 after 5% formalin injection. **(A–C)** p-p38 immunoreactivity on the ipsilateral side of the dorsal spinal cord from naïve, vehicle-, and simvastatin-treated animals (scale bars: 100 μm). **(D** and **E)** Representative bands and quantification of Western blot analysis showing that simvastatin suppressed the increased level of p-p38. *$P < .05$, as compared with the vehicle group (n = 6). **(F–I)** Representative bands and quantification of p-ERK and p-JNK Western blot; there was no statistically significant difference with and without the simvastatin treatment (n = 6).

an increase of Iba-1 (Figs 3A and 3B) and CD11 b expression (Figs 3F and 3G). In the presence of simvastatin (10 μg), microglial activation was significantly attenuated as observed both in the quantity of Iba-1 and CD11 b immunolabeling and in the reduction in morphologic changes in microglia (Figs 3C and 3H). Similarly, quantitative protein analysis using Western blot showed reductions after simvastatin administration (Figs 3D, 3E, 3I, and 3J).

Simvastatin Inhibited Formalin-Induced Spinal Microglial p-p38 MAPK Activation, But Not p-ERK or p-JNK Activation

In our previous study, peripheral formalin injection activated spinal microglial p38 MAPK pathway contributing to the increased nociceptive behaviors.[24] To test microglial activity of MAPK cell signaling pathway

in the presence of simvastatin after formalin injection, we investigated p-p38, p-ERK, and p-JNK activation in the spinal cord with and without simvastatin. Both immunohistochemical staining and Western blot analyses showed that formalin injection into the plantar hind paw resulted in an increase of p-p38 expression that was significantly attenuated by simvastatin (10 μg) treatment (Figs 4A–E). However, the expression of p-ERK and p-JNK was not changed by simvastatin treatment (Figs 4F–I). Consistent with our previous publication,[24] peripheral formalin injection did not produce significant changes of p-ERK or p-JNK expression.

Peripheral Formalin Injection Induced an Increase in RhoA Expression in Spinal Cord Microglia

Immunolabeling was used to observe RhoA expression in the spinal cord. At day 7 after formalin injection, RhoA expression in the ipsilateral side of the spinal cord dorsal horn was much greater than on the contralateral side or in naïve rats (Figs 5A and 5B). Using double labeling, we noted that RhoA was expressed in both microglia and astrocytes, but mainly was colocalized with activated microglia (Figs 5C–E).

Simvastatin Blocked the Formalin-Induced Increase of Membrane Translocation of RhoA

RhoA exerts biological function through translocation from cytoplasm to membrane. To explore the effect of simvastatin on the spinal RhoA in our study, we detected both membrane and cytosolic RhoA in the spinal cord after 7 days of simvastatin treatment, and calculated membrane/cytosol ratio of RhoA (RhoA translocation ratio). In the naïve group, RhoA was predominantly localized in cytosolic fraction. On day 7 after formalin injection, we observed an increase in the RhoA translocation ratio (Fig 6), indicating that formalin injection injury increased the translocation of RhoA from cytoplasm to membrane. The formalin-induced membrane translocation of RhoA was significantly blocked by intrathecal treatment with simvastatin (10 μg) (Fig 6).

Discussion

Several previous studies have demonstrated that statins attenuate microglial inflammatory responses in different models such as traumatic brain injury and beta-amyloid-induced inflammation.[7,9,23] The statins exert anti-inflammatory action by way of inhibiting the activation of microglia and astrocytes after brain

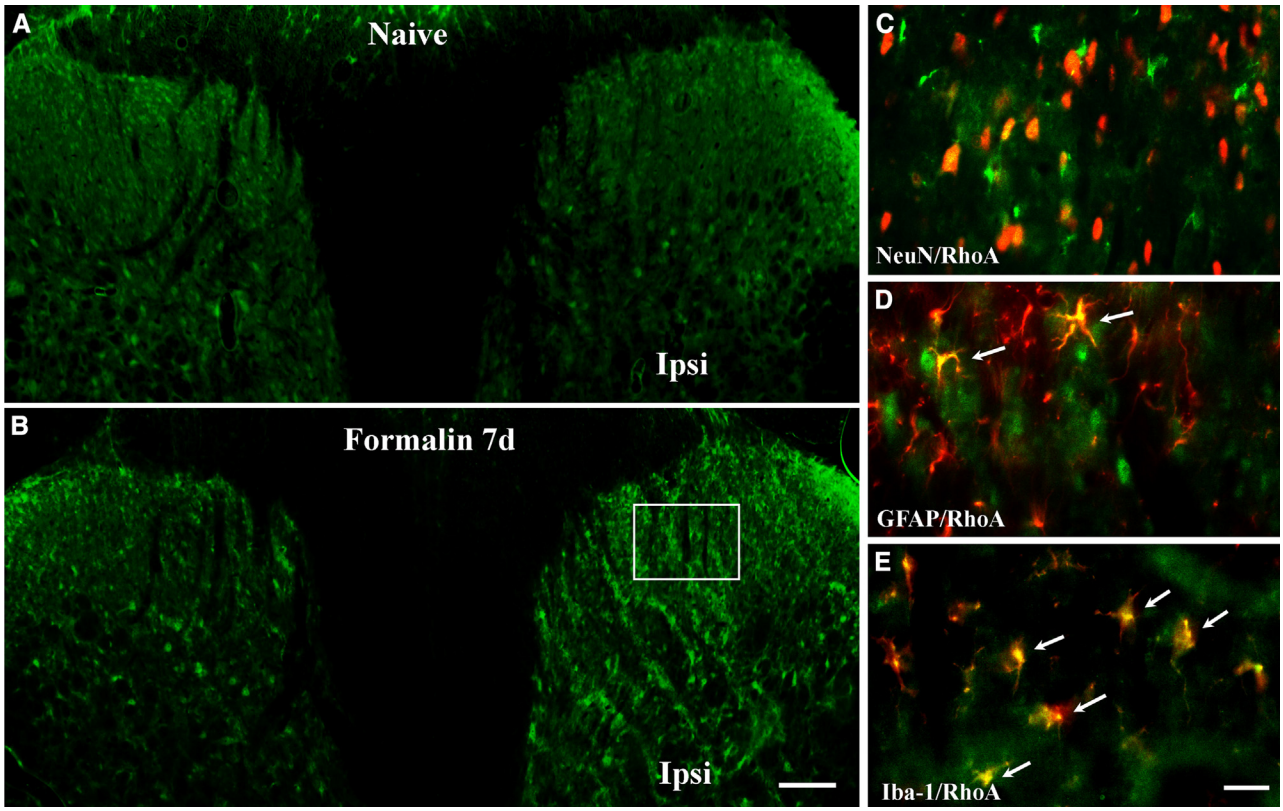

Figure 5. Peripheral formalin injection induced RhoA activation in the lumbar spinal dorsal horn. **(A** and **B)** Representative immunostaining images of RhoA expression in naïve and formalin-injected animals at day 7 after injection (scale bar: 100 μm). **(C–E)** Merged images of double immunofluorescent labels of RhoA (green) with markers of neurons (red, NeuN), astrocytes (red, GFAP), and microglia (red, Iba-1); arrows indicated coexpressed astrocyte **(D)** and microglia cells **(E)** (scale bars: 20 μm).

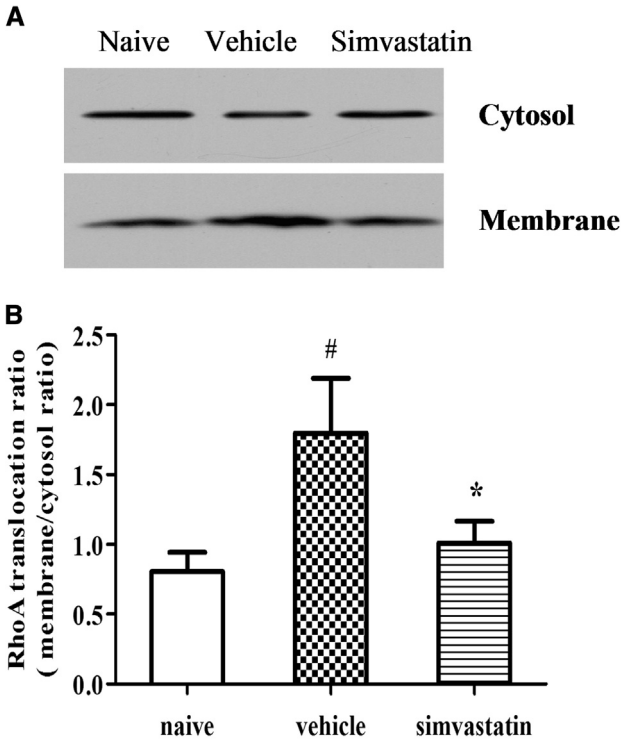

Figure 6. The effect of i.t. injection of simvastatin on the subcellular localization of RhoA in the ipsilateral spinal cord at day 7 after 5% formalin injection. **(A)** The representative bands of RhoA expression in membrane and cytosol after formalin injection with and without simvastatin. **(B)** The quantification of membrane/cytosol ratio of RhoA (RhoA translocation ratio) in each group, with simvastatin reversing the upregulation of RhoA translocation ratio. #$P < .05$ compared with the naïve group. *$P < .05$ compared with the vehicle group (n = 4).

insults.[23] However, the mechanisms involved in the analgesic effects of statins are not yet fully understood.

In the present study, we observed that intrathecal pretreatment with simvastatin caused a conspicuous pain-alleviating effect on the second phase of spontaneous nociceptive response to formalin. Similar findings were already reported by others.[13,30] In addition, continued application of simvastatin once a day for 7 days alleviated the persistent mechanical hyperalgesia normally seen 7 days after formalin injection. Spinal microglial activation normally caused by formalin injection was also suppressed by simvastatin injection. The morphologic activation observed using Iba-1 and CD11 b and the increases in phophorylated-p38 MAPK were all reduced by simvastatin. Finally, we also found for the first time that formalin injection produced an increase in membrane RhoA translocation ratio, and this effect was also reduced by simvastatin. Immunofluorescence also confirmed that RhoA-positive cells coexpressed Iba-1, suggesting that RhoA is expressed by microglia. These findings together strongly suggest that simvastatin exerts analgesic effects on formalin-induced nociceptive behaviors by attenuating spinal microglial activation via molecular mechanisms that include the interruption of microglial RhoA translocation and p38 MAPK activation.

It is well known that statins have cholesterol-independent effects that include the prevention of biological activities downstream from L-mevalonate.[2,15] The mevalonate pathway produces not only cholesterol but also various isoprenoids such as farnesyl pyrophosphate and geranylgeranylpyrophosphate.[16] Both of these are important mediators involved in the modification of a large number of proteins including small molecular G proteins. The small G proteins are suggested to be essential elements in inflammatory signaling cascades.[25] Intrathecal administration of exogenous mevalonate dose-dependently decreased the paw-withdrawal latencies for thermal stimulation and increased the amount of geranylgeranylated (one pattern of isoprenylation) RhoA in the spinal cord.[29]

Isoprenylation, known as lipid modification, is essential for the translocation of small molecular G proteins from cytosol to plasma membrane and for the activation of subsequent intracellular signaling. RhoA, a small GTPase, is a member of a family of small molecular G proteins, which are involved in many cellular functions including cytoskeletal rearrangement, cell motility, phagocytosis, intracellular trafficking, transcriptional regulation, and cell growth and development.[40,44] RhoA translocation is crucial for signaling downstream to the molecular Rho kinase (ROCK).[17] Several reports have shown that the RhoA/ROCK signaling pathway plays important roles in the development and/or maintenance of chronic pain.[19,28,31,41] The spinal cord of streptozotocin-treated diabetic mice showed increased membrane-bound RhoA; treatment with the RhoA inhibitor exoenzyme C3, *Clostridium botulinum*, and the ROCK inhibitor Y27632 attenuated thermal hyperalgesia and mechanical allodynia in diabetic mice. Moreover, daily treatment with simvastatin attenuated all of these changes in diabetic mice.[28] Intrathecal treatment with the ROCK inhibitor Y27632 attenuates cold hyperalgesia in C7/8 rhizotomy.[31]

In the present study, following formalin injection, upregulation of RhoA expression level was observed throughout the spinal cord dorsal horn, mainly in the activated microglia. In the membrane-localized RhoA extraction experiment, we found that peripheral formalin injection caused the increase of RhoA translocation ratio that could be reversed by simvastatin treatment. This finding suggested that simvastatin interrupted formalin-induced RhoA activity within the spinal microglia. RhoA may also be expressed in neurons and astrocytes, as well as microglia. Although RhoA was coexpressed with GFAP, we did not further quantify RhoA-positive cells in different types of cells because we did not find astrocyte activation using GFAP staining in formalin pain model (data not shown).

MAPKs in microglia have been demonstrated to play important roles in pathological pain conditions. p38 MAPK signaling pathway was involved in the development of formalin-induced nociceptive behaviors; intrathecal administration of p38 inhibitor SB203580 not only inhibited the early acute spontaneous nociceptive behaviors but also reversed the formalin injury–induced long-term persistent mechanical hyperalgesia.[24] In the present study, compared to the saline-injected vehicle group, simvastatin treatment distinctly attenuated

microglial Iba-1 and CD11 b expression in the spinal cord, in addition to attenuating p-p38 expression following formalin injection. As in our previous report that peripheral formalin injection did not produce increases of p-ERK and p-JNK expression,[24] we did not find significant changes of p-ERK and p-JNK expression with and without the treatment of simvastatin. Some studies have implied a molecular link between the inhibitory effect of statins and the intracellular pathway p38 MAPK and Rho/ROCK kinases. Statins have been shown to regulate p38 MAPK through the inhibition of Rho activation in a variety of cell types[14,33,42] and thus exert their pleiotropic effects through inactivation of the Rho-MAPK pathway.[4,35,37] Although we did not test p-p38 and RhoA expression at an early time point in the present study, our previous study suggested that peripheral formalin induced 2 stages of spinal microglial activation. The p38 MAPK was also rapidly activated in the spinal microglia minutes after injection, and the activation persisted for 1 hour (phase II of the nociceptive behavior), in addition to a secondary increase that was detected for days and was maximal at day 7 postinjection.[24] Simvastatin may also decrease formalin-induced phase II spontaneous nociceptive behaviors through the pathway of inhibiting microglial p38 activation.

In summary, simvastatin exerted an inhibitory effect on formalin-induced nociceptive behaviors and spinal microglial activation. Simvastatin inhibited formalin-induced spinal microglial p38 activation but not ERK and JNK activation. Further investigation revealed that formalin injection induced an increase in membrane RhoA translocation ratio in the spinal activated microglia, and this effect was reversed by simvastatin. Our novel findings suggested that simvastatin caused inhibition of formalin-induced nociceptive responses, at least in part, through inhibiting microglial RhoA and p38 MAPK activation. Inactivation of the RhoA-p38 signaling pathway may be a pharmacologic target for treating microglia-directed CNS inflammation and chronic pain conditions.

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